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WRN, the protein deficient in Werner syndrome, plays a critical structural role in optimizing DNA repair

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Summary

Werner syndrome (WS) predisposes patients to cancer and premature aging, owing to mutations in *WRN*. The WRN protein is a RECQ-like helicase and is thought to participate in DNA double-strand break (DSB) repair by non-homologous end joining (NHEJ) or homologous recombination (HR). It has been previously shown that non-homologous DNA ends develop extensive deletions during repair in WS cells, and that this WS phenotype was complemented by wild-type (wt) WRN. WRN possesses both 3′ → 5′ exonuclease and 3′ → 5′ helicase activities. To determine the relative contributions of each of these distinct enzymatic activities to DSB repair, we examined NHEJ and HR in WS cells (WRN–/–) complemented with either wtWRN, exonuclease-defective WRN (E–), helicase-defective WRN (H–) or exonuclease/helicase-defective WRN (E–H–). The single E– and H– mutants each partially complemented the NHEJ abnormality of WRN–/– cells. Strikingly, the E–H– double mutant complemented the WS deficiency nearly as efficiently as did wtWRN. Similarly, the double mutant complemented the moderate HR deficiency of WS cells nearly as well as did wtWRN, whereas the E– and H– single mutants increased HR to levels higher than those restored by either E–H– or wtWRN. These results suggest that balanced exonuclease and helicase activities of WRN are required for optimal HR. Moreover, WRN appears to play a structural role, independent of its enzymatic activities, in optimizing HR and efficient NHEJ repair. Another human RECQ helicase, BLM, suppressed HR but had little or no effect on NHEJ, suggesting that mammalian RECQ helicases have distinct functions that can finely regulate recombination events.

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Introduction

Individuals with Werner syndrome (WS) prematurely develop an aged appearance and many common age-related disorders, beginning in early adulthood (Epstein *et al*., 1966; Tollefsbol & Cohen, 1984; Belmaaza & Chartrand, 1994; Goto, 1997). Death generally occurs in the fifth decade of life, primarily from cancer and cardiovascular disease. Cells from WS individuals have a short replicative lifespan in culture, and exhibit genomic instability characterized by chromosomal variegated translocation mosaicism (Salk *et al*., 1981). *WRN*, the gene defective in WS, encodes a protein that is homologous to the *E. coli* RECQ helicase, which plays an important role in recombinational repair of DNA damage (Yu *et al*., 1996). In addition to WRN, four other RECQ-like proteins have been identified in humans, including RECQL1, BLM (which is defective in Bloom syndrome), RTS (which is defective in Rothmund–Thomson syndrome) and RECQ5 (Oshima, 2000). It is not known whether these five mammalian RECQ-like helicases mediate overlapping or unique functions within cells.

RECQ proteins are 3′ → 5′ ATP-dependent helicases (Gray *et al*., 1997; Suzuki *et al*., 1997). WRN also exhibits intrinsic 3′ → 5′ exonuclease activity (Huang *et al*., 1998), which is not found in the other RECQ helicases. The WRN helicase unwinds unusual DNA structures, such as double-stranded DNA with mismatched tails (Suzuki *et al*., 1997), bimolecular G4 quartets (Fry & Loeb, 1999) and Holliday junctions (Constantinou *et al*., 2000). The WRN exonuclease preferentially degrades double-stranded DNA with mismatched bubbles and staggered ends (Huang *et al*., 2000; Shen & Loeb, 2000). These abnormal structures can occur physiologically, or can be accidentally generated by DNA damage, during DNA repair or recombination, suggesting that WRN plays a role in recombinational repair (Oshima, 2000). Indeed, WRN physically interacts with components of the two major systems for recombinational repair: non-homologous end joining (NHEJ, or illegitimate recombination) and homologous recombination (HR). NHEJ imprecisely joins the ends of double-stranded DNA breaks (DSBs). NHEJ requires DNA-dependent protein kinase (DNA-PK), which consists of a catalytic subunit (DNA-PKcs) and a DNA end-binding heterodimer (Ku), ligase IV, and XRCC4. WRN interacts with both Ku and DNA-PKcs (Cooper *et al*., 2000; Li & Comai, 2000), and is a substrate for DNA-PKcs (Yannone *et al*., 2001).

In vitro studies suggest that assembly of DNA-PK and WRN at DNA ends allows DNA-PKcs to phosphorylate WRN, thereby stimulating WRN enzymatic activity and facilitating efficient

processing of DSBs prior to ligation (Yannone *et al*., 2001). In contrast to NHEJ, HR repair is relatively error-free, as it uses homologous sequences to process and repair DSBs. WRN interacts with hRAD51, a molecule similar to bacterial RecA that promotes homologous pairing and strand exchange (Sakamoto *et al*., 2001). In human fibroblasts, WRN localizes predominantly to nucleoli; however, when cells are exposed to genotoxic agents, WRN translocates to the nucleoplasm, suggesting that it may play a role in repairing DNA lesions induced by NHEJ and/or HR (Gray *et al*., 1998; Sakamoto *et al*., 2001).

In contrast to WS, Bloom syndrome (BS) is associated with severe pre- and postnatal growth retardation, a high incidence of cancer, and death in the second or third decade of life, primarily from cancer (Ellis & German, 1996). Cells from BS individuals are genomically unstable, and typically accumulate sister chromatid exchanges, which is a hallmark of elevated HR (German, 1995). The BLM helicase, like WRN, unwinds G4 quartets and Holliday junctions (Sun *et al*., 1998), and physically interacts with hRAD51 (Bischof *et al*., 2001). However, BLM is primarily localized within a subnuclear region termed the PML body, which contains the promyelocytic leukaemia (PML) tumour suppressor protein and hRAD51 (Ishov *et al*., 1999; Zhong *et al*., 1999). Moreover, whereas WRN is expressed at similar levels throughout the cell cycle (Kawabe *et al*., 2000), BLM is expressed predominantly during the S and G2 phases, when HR is favoured (Kawabe *et al*., 2000). In response to DNA damage, BLM co-localizes with hRAD51 to sites of presumptive DSB repair (Kawabe *et al*., 2000), supporting the hypothesis that it plays a role in HR DNA repair (Chakraverty & Hickson, 1999).

We have shown that linear DNA undergoes extensive deletion of non-homologous ends during repair in WS cells, and that this phenotype is complemented by expression of wild-type WRN in WS cells (Oshima *et al*., 2002). We speculated that the WRN helicase may unwind DSBs to facilitate a search for local microhomology, and/or that the WRN exonuclease may trim the DSB ends, in preparation for completion of repair by XRCC4 and ligase IV. Because WRN has relatively weak enzymatic exonuclease and helicase activities, we suggested it might out-compete more robust enzymes, thereby limiting the extent of unwinding and degradation. Alternatively, WRN may stabilize broken DNA ends, similar to the proposed role of the Ku protein, which interacts with WRN. In this study, we investigated how the exonuclease and helicase activities of WRN, as opposed to its physical presence, regulate NHEJ and HR repair. In addition, we investigated the role of BLM, which was shown to play a more dominant role than WRN in HR (Imamura *et al*., 2002), in these repair processes.

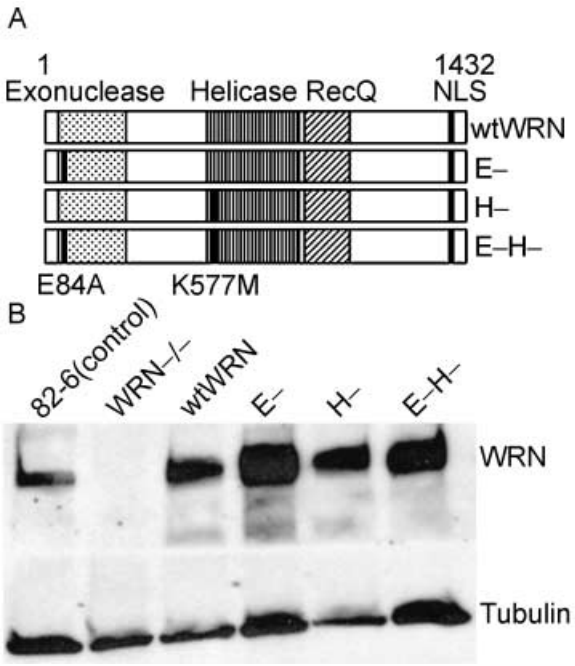

Fig. 1 Structure of wild-type and mutant WRN proteins and expression in WS cells. (A) Schematic representation of the structures of wild-type WRN (wtWRN), the exonuclease mutant E84A (E–), the helicase mutant K577M (H–) and the exonuclease and helicase double mutant E84A and K577M (E–H–). Diagrams show the sites of these mutations with respect to the exonuclease, helicase, RECQ consensus and nuclear localization signal (NLS) domains of WRN, with a total length of 1432 amino acids. (B) The wtWRN (E–), (H–) and (E–H–) constructs shown in panel A were expressed in hTERT-immortalized WS (WRN–/–) fibroblasts. Total cell lysates were subjected to Western blot analysis to determine WRN levels in 82-6hTERT (control) normal cells, WRN–/– cells and WRN–/– cells expressing either the wtWRN, E–, H– or E–H– constructs, as indicated. The upper panel shows WRN immunoreactivity and the lower panel shows tubulin protein levels, as a control.

Results

Expression of wild-type and mutant WRN in hTERT-immortalized WS fibroblasts

To eliminate variation due to differences in genetic background, which might confound the outcome of cell-based assays, we constructed a series of isogenic WS cell lines. The parent cells were human fibroblasts isolated from a WS patient, which we immortalized using a retrovirus carrying the catalytic subunit of human telomerase (73-26hTERT) (WRN–/– cells) (Oshima *et al*., 2002). We then used different retroviruses (Oshima *et al*., 2002) to express either wild-type or one of the three mutant WRN proteins in WRN–/– cells (Fig. 1A). Because WRN has two enzymatic activities (exonuclease and helicase), we also generated H– cells (expressing WRN carrying a point mutation that abolishes helicase activity), E– cells (expressing WRN carrying a point mutation that abolishes exonuclease activity) and E–H– cells (expressing WRN carrying both mutations). As a control, uncomplemented WRN–/– cells were infected with an insertless retrovirus.

To confirm that the fibroblasts expressed WRN, protein lysates were subjected to Western blotting using a WRN C-terminal specific antibody (Fig. 1B). WRN immunoreactivity was detectable as a specific 180-kDa band in all cell lines except WRN–/– cells. All four WRN-complemented cells (wtWRN, WRN E–, WRN H–, WRN E–H–) expressed WRN protein at levels similar to that of the control cell line, 82-6hTERT, when normalized to

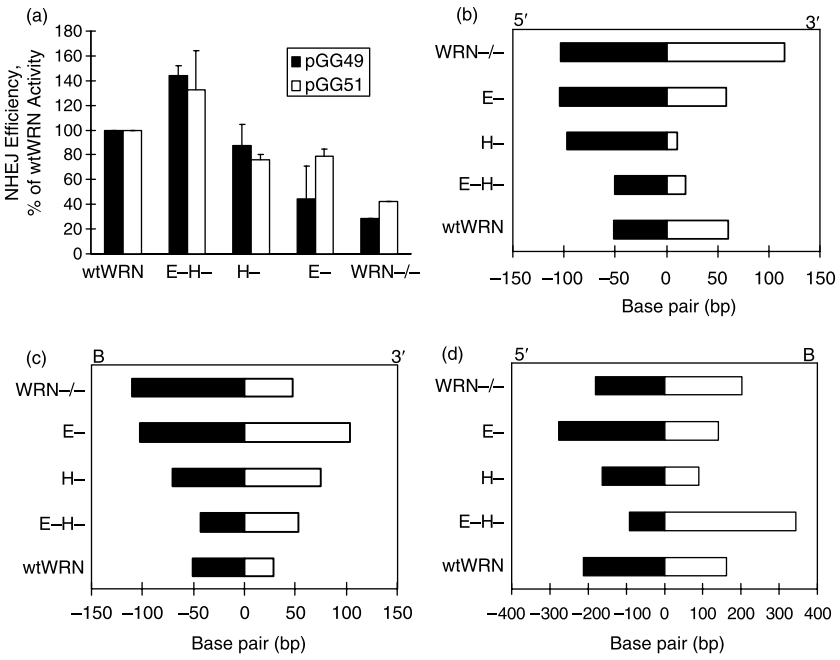

Fig. 2 Analysis of NHEJ in WRN–/– and WRN-complemented cells. (A) V(D)J recombination assay. The efficiency of NHEJ was assessed by the V(D)J recombination assay. The GG49 plasmid was used to assay joining at the recombination signal side of the DSB, and pGG51 was used to assay joining at the coding sequence side of the DSB, as described in Experimental procedures. The data were normalized to the efficiency of NHEJ in wtWRN cells, and are expressed as the average percentage of wtWRN activity ± standard deviation from six independent experiments, each performed in duplicate transfections. (B–D) pBS joining assays. (B) The extent of deletion in linearized plasmids with 5′ and 3′ protruding ends. (C) The extent of deletion in linearized plasmids with blunt (B) and 3′ protruding ends. (D) The extent of deletion in linearized plasmids with 5′ protruding and blunt ends. The *x*-axis indicates the number of bp deleted, with the site of linearization designated as 0. Data are expressed as the average deletion size from a minimum of 10 rescued plasmids.

tubulin immunoreactivity levels. These results indicate that all the WRN-complementing constructs expressed the expected protein in WRN–/– cells, and at levels similar to that of endogenous WRN in normal cells. Subsequent cell-based assays were performed using these isogenic WRN–/– and WRN-complemented cells.

Effects of single and double WRN mutants in V(D)J recombination assay

The V(D)J joining assay has been widely used to assess NHEJ activity in a variety of cells, including fibroblasts from DNA-PKcs and Ku knockout mice (Taccioli & Alt, 1995; Gu *et al*., 1997a,b). The assay takes advantage of processes that generate immunoglobulin and T-cell receptor variable region diversity by V(D)J recombination (Tonegawa, 1983). Recombination is initiated by the introduction of DSBs between V, (D) or J coding sequences and their flanking recombination signal sequences. The initiating DSB generates blunt ends at the signal sequence, and hairpin structures at the coding ends, which are carried out by the tissue-specific RAG-1 and RAG-2 proteins (Schatz *et al*., 1989; Oettinger *et al*., 1990). Completion of V(D)J recombination is carried out by factors that are ubiquitously expressed across nearly all cell types, and these factors are also involved in DSB repair by NHEJ (Taccioli & Alt, 1995). Thus, the efficiency of NHEJ recombination can be measured in any cell type if RAG-1 and RAG-2 are provided with a test plasmid containing V, (D) and J sequences. WRN–/– and WRN-complemented cells were transfected with RAG-1 and -2 expression vectors and the test V(D)J plasmid. The appearance of chloramphenicol (Cm) resistance in plasmids rescued from transfected cells indicated that DSBs were successfully rejoined. The pGG49 plasmid reported joining of coding sequences, whereas pGG51 reported signal sequence joining.

We measured the frequency of V(D)J recombination (the number of Cm-resistant colonies as a percentage of the total number of colonies) from duplicate transfections in each assay. The activity of WRN–/–, H–, E– and E–H– cells was normalized to the activity of wtWRN cells. Results from six independent assays are shown in Fig. 2(A). The frequency of coding joining and signal joining in WRN–/– cells was 29% and 43%, respectively, of that observed in wtWRN cells. Thus, WRN was essential for efficient NHEJ in this assay. E– cells exhibited 44% and 79% of the coding and signal joining frequencies observed in wtWRN cells, whereas H– cells showed 88% and 76% of these frequencies, respectively. Thus, mutations that inactivate either the exonuclease or the helicase activities of WRN reduced the efficiency of NHEJ to a level intermediate between that of WRN–/– and wtWRN cells, indicating that both enzymatic activities of WRN contribute to optimal NHEJ. Surprisingly, the E–H– double mutant cells exhibited somewhat more efficient NHEJ than did wtWRN cells, with 144% and 133% coding and signal joining frequencies, respectively. This finding raises the possibility that WRN has a structural function in NHEJ, such as regulating interactions among other proteins.

To investigate the nature of NHEJ in WRN–/– and WRN-complemented cells, the recombination region of the test plasmids was sequenced. We sequenced a total of 126 rescued recombined plasmids, and determined the extent of the deletions that occurred as a result of NHEJ. All deletions were relatively small (most < 10 bp), regardless of WRN status. This is an apparent contradiction to our previous observation that WRN–/– cells induce larger deletions than wtWRN cells during NHEJ (27). However, it is possible that plasmids with large deletions were generated in the V(D)J assay, but that these deletions extended into the *Cm* gene, thereby precluding their rescue. Thus, reduced

V(D)J rejoining in this assay may reflect, at least in part, extensive NHEJ-generated deletions.

pBluescript joining assay confirms results of the V(D)J assay

To test the hypothesis that reduced V(D)J rejoining in WRN–/–, H– and E– cells was due primarily to extensive deletion, rather than the inability to ligate DSB ends in the absence of fully functional WRN, we performed a pBluescript joining assay (pBS joining assay) in the same cell lines. In this assay, transfected plasmids are first linearized with restriction enzymes that create ends with defined characteristics (blunt, 3′ or 5′ overhangs), and the average length of deletion at both ends of the break site reflects the ability of cells to preserve the ends. We previously noted that the extent of the deletion in a given cell line was related to the size of the linear test plasmid (Oshima *et al*., 2002).

The pBS joining assay confirmed that WRN–/– cells create larger deletions than wtWRN cells during NHEJ, at least when the break site contained a 3′ overhang (Fig. 2) (Oshima *et al*., 2002). For WRN–/– cells vs. wtWRN cells, the total average deletion sizes were: 218 vs. 112 bp for plasmids with 5′ and 3′ protruding ends; 157 vs. 80 bp for plasmids with blunt and 3′ protruding ends; and 383 vs. 384 bp (no difference) for plasmids with 5′ protruding and blunt ends. In general, but with some exceptions, H– and E– cells exhibited an intermediate phenotype (between WRN–/– and wtWRN cells) with regard to deletion size. Total average deletion sizes in H– and E– cells were 108 and 162 bp for plasmids with 5′ and 3′ protruding ends; 146 and 206 bp for plasmids with blunt and 3′ protruding ends; and 253 and 419 bp for plasmids with 5′ protruding and blunt ends. Cells expressing the double mutant WRN (E–H–) behaved more like wtWRN cells than any of the other cells, similar to what was observed in the V(D)J recombination assay. Moreover, in some cases, E–H– cells preserved broken DNA ends somewhat better than did wtWRN in the pBS joining assay. The average deletion sizes in E–H– cells were 69 bp for plasmids with 5′ and 3′ protruding ends, 96 bp for plasmids with blunt and 3′ protruding ends, and 436 bp for plasmids with 5′ protruding and blunt ends.

In some cases, E– or H– cells generated larger deletions than did WRN–/– cells. Because the pBS joining assay samples only a limited number of variations, one plasmid with an unusually large deletion can skew the average value. Nevertheless, the trend observed in the pBS joining assay indicated that plasmids rescued from WRN–/–, H– and E– cells had larger deletions than those rescued from wtWRN or the double mutant E–H– cells. This supports the hypothesis that the differences observed in the V(D)J assay were due to differences in the ability of the various constructs to preserve DNA ends during NHEJ.

WRN slightly enhances HR repair

DNA DSBs can also be repaired by homologous recombination (HR). We therefore investigated what role, if any, WRN plays in HR repair. Our assay used a plasmid, pCMS-homo, which carries genes for green and yellow fluorescent proteins (GFP and EYFP), with a series of restriction sites separating a pair of homologous EYFP sequences. The plasmid was linearized by cutting at two restriction sites to generate incompatible ends, and then transfected into cells. All transfected cells will express GFP fluorescence. By contrast, cells will express EYFP fluorescence only if the plasmid is repaired by HR. The frequency of HR repair was calculated as the ratio of cells expressing EYFP + GFP vs. cells expressing GFP only, normalized to the frequency of HR repair of wtWRN cells (average of three independent experiments per data point) (Fig. 3). WRN–/– cells had the lowest HR repair frequency (85%) among all cells tested. E–H– cells were most similar to wtWRN cells (110%), whereas E– and H– cells showed slightly higher rates of HR repair (134% and 141%, respectively), as compared with wtWRN cells. The effects of WRN status on HR repair were modest, but statistically significant ($P < 0.01$ for wtWRN vs. WRN–/–, wtWRN vs. H– and wtWRN vs. E–, $P = 0.97$ for wtWRN vs. E–H–, and $P = 0.42$ for H– vs. E–). Taken at face value, the results suggest that WRN slightly enhances HR repair. Because E–H– cells were as proficient as wtWRN cells, this enhancement may not require WRN enzymatic activities. However, single WRN mutations (E– or H– cells) slightly stimulated the HR repair frequency, thereby raising the possibility that unbalanced WRN activity (helicase activity in the absence of exonuclease activity, or vice versa) stimulates other proteins involved in HR repair.

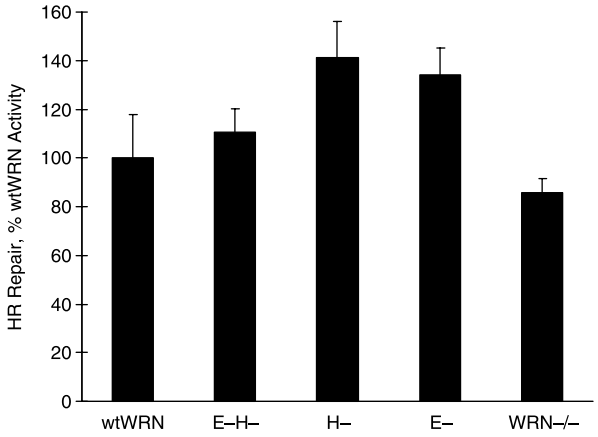

Fig. 3 pCMS-homo HR repair assays. HR repair frequencies were measured as described in Experimental procedures. Data are expressed as the average percentage of HR repair observed in wtWRN cells ± standard deviation from two independent experiments, each performed in triplicate transfections.

BLM suppresses HR repair

BLM, another RECQ-like helicase, has also been implicated in HR repair. Specifically, the increased rate of sister chromatid exchange in BS cells suggests that BLM suppresses HR during DSB repair (German, 1995). We therefore tested the effects of BLM on HR repair, to validate our assay. First, we generated isogenic

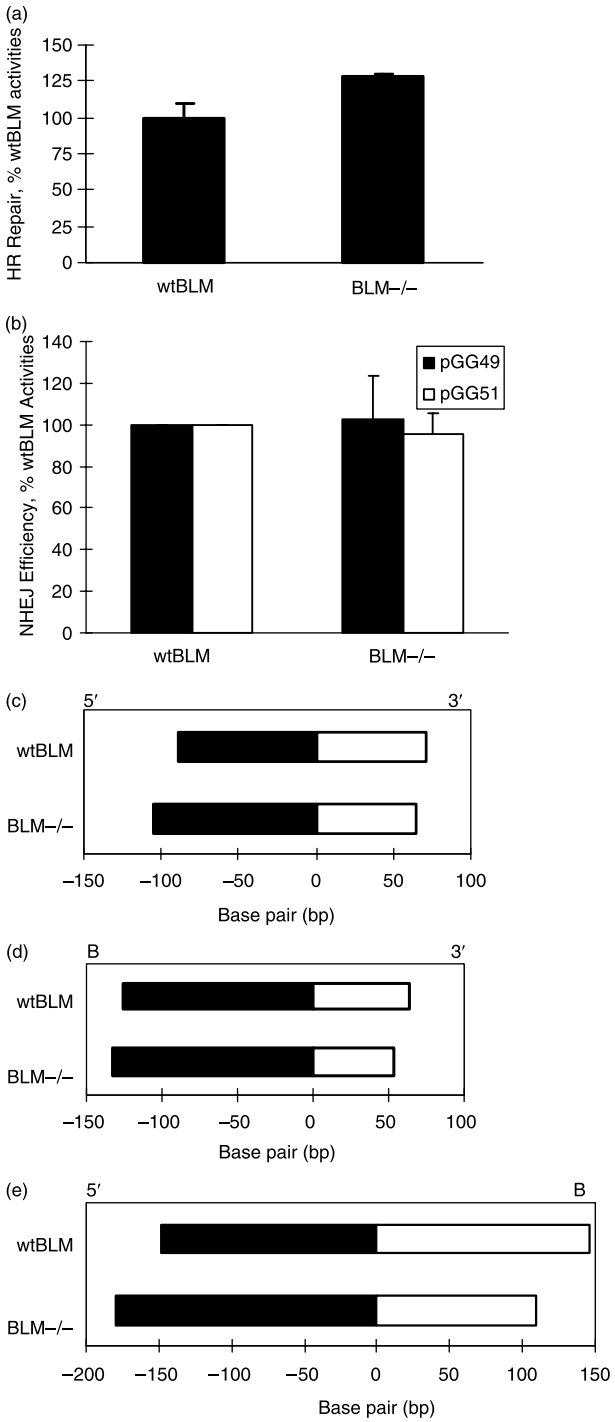

Fig. 4 NHEJ and HR repair assays in BLM–/– and complemented wtBLM cells. (A) The pCMS-homo repair assay was used to determine HR repair frequency in wtBLM and BLM–/– cells. The repair frequency of wtBLM–/– cells is expressed as the average percentage of that in wtBLM cells ± standard deviation from two independent experiments, each performed in triplicate transfections. (B) V(D)J recombination assay using BLM–/– and wtBLM cells. Experiments and analysis were performed as described in Fig. 2(A). (C–E) pBS joining assays using BLM–/– and wtBLM cells. (C) The extent of deletion in linearized plasmids with 5′ and 3′ protruding ends. (D) The extent of deletion in linearized plasmids with blunt (B) and 3′ protruding ends. (E) The extent of deletion in linearized plasmids with 5′ protruding and blunt ends, from the average deletion size in a minimum of 10 rescued plasmids. The *x*-axis indicates the number of bp deleted, with the site of linearization designated as 0.

BS cells using strategies similar to those employed for WS cells. Parental fibroblasts, HG2654 cells, were immortalized with hTERT (BLM–/– cells), and then complemented by introducing a wild-type BLM cDNA (wtBLM cells) (A. Davalos & J. Campisi, unpubl. data). The HR repair frequency in BLM–/– cells was 128% of that in wtBLM cells ($P < 0.05$) (Fig. 4A), verifying that BLM suppresses HR during DSB repair and validating the assay.

BLM has no significant activity in V(D)J and pBS NHEJ assays

We next asked whether BLM plays a role in NHEJ. In the V(D)J recombination assay (Fig. 4B), BLM–/– cells were essentially as active as wtBLM cells (103% and 95%, for pGG49 and pGG51, respectively), indicating that BLM status does not affect the efficiency of NHEJ. In the pBS joining assay, BLM–/– and wtBLM cells showed average deletion sizes of 169 and 159 bp for plasmids with 5′ and 3′ protruding ends (Fig. 4C), 186 and 190 bp for plasmids with blunt and 3′ protruding ends (Fig. 4D), and 288 and 293 bp for plasmids with 5′ protruding and blunt ends (Fig. 4E). These results are consistent with the hypothesis that large deletions at the DSB ends reduce the apparent efficiency of NHEJ in the V(D)J recombination assay. It should be noted that the overall V(D)J recombination frequencies in BS lines were lower than those in WS lines, and the average deletion sizes in BS lines were larger than those in WS lines, probably due to differences in the genetic backgrounds of the parental cells. These differences emphasize the importance of comparing isogenic cells for these types of studies. We concluded that BLM has little or no effect on NHEJ *in vivo*.

In summary, the loss of WRN function decreased HR activity by 15% (Fig. 3), whereas loss of BLM function increased HR activity by 28% (Fig. 4A). These findings indicate that, although WRN and BLM are both RECQ helicases, they appear to play distinct roles in recombinational repair of DSBs, as illustrated schematically in Fig. 5. It appears that WRN preserves the ends

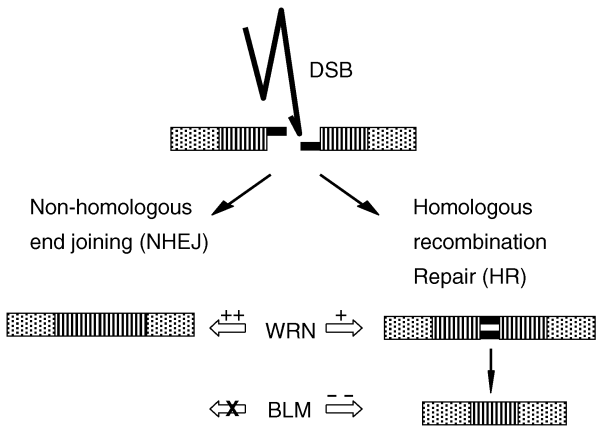

Fig. 5 Proposed role of WRN and BLM in NHEJ and HR repair of DNA DSBs. DSBs are repaired through either the NHEJ or the HR repair pathways. WRN enhances NHEJ and, to a lesser extent, HR repair. BLM suppresses HR, but has no effect on NHEJ.

of non-homologous DNA at DSBs, whereas BLM seems to be inactive in this process. In contrast, WRN appears to stimulate HR modestly during DSB repair, whereas BLM suppresses HR during DSB repair.

Discussion

WRN and *BLM* both encode RECQ-like proteins that have been implicated in DSB repair and recombination. We confirmed that an absence of WRN causes large deletions during the repair of non-homologous DSB ends using two different assays for NHEJ. Our results also indicate that the reduced frequency of V(D)J recombination in WRN–/– cells, as compared with wtWRN cells, is due primarily to large deletions generated at the break sites of the test plasmids.

The V(D)J recombination and NHEJ assays consistently showed that WRN mutations that selectively abolish either the exonuclease or the helicase activity of the enzyme only partially complement the large deletion phenotype of WRN–/– cells. These findings alone suggest that both exonuclease and helicase functions are necessary for optimal NHEJ. However, the double WRN mutant was able to complement the deletion phenotype with equal efficiency as wild-type WRN. This observation suggests that balanced WRN exonuclease and helicase activities, with either both active or both inactive, may be more important for the control of DSB end processing than the presence of either activity alone.

The excessive deletions that occur during NHEJ in WRN–/– cells suggest that other, more active, exonuclease(s) or helicase(s) can replace WRN when this protein is absent. Because helicases and nucleases function with specific directionality, WRN is predicted to move along DNA in opposite directions when it is unwinding DNA (helicase activity) vs. when it is degrading DNA (exonuclease activity) (Oshima, 2000). Thus, the deletions observed in E– cells might be due to activity of competing exonucleases, or to unopposed helicase activity within the same WRN molecule. Similarly, deletions that occur in H– cells could be due to extensive unwinding by competing helicases, or unopposed exonuclease activity. When both exonuclease and helicase activities are abolished, WRN may be unable to move along the broken DNA and may thereby minimize degradation prior to ligation. In this scenario, the inactive WRN could structurally hinder competing enzymes. Thus, our results raise the possibility that WRN plays a previously unknown structural role in protecting DSB ends during NHEJ.

It has been well established that both enzymatic activities of WRN can be modulated by interactions with other proteins *in vitro*. WRN exonuclease activity is suppressed by interaction with p53 (Brosh *et al*., 2001) or BLM (von Kobbe *et al*., 2002), and stimulated by interaction with Ku70/80 (Li & Comai, 2001) or phosphorylation (Karmakar *et al*., 2002). WRN helicase activity is stimulated by interaction with p53 (Yang *et al*., 2002), replication protein A (RPA) (Shen *et al*., 1998), telomere repeat binding factor 2 (TRF2) (Opresko *et al*., 2002) and phosphorylation (Karmakar *et al*., 2002). Given that WRN can reside in a large multiprotein complex (Brosh & Bohr, 2002), it is likely that its enzymatic activities are regulated *in vivo* by phosphorylation and by interactions with other proteins. However, we cannot rule out the possibility that the point mutations we generated have other effects in addition to abolishing exonuclease or helicase activity – for example, abolishing interactions with other molecules *in vivo*. Nevertheless, the results of our V(D)J and NHEJ assays strongly suggest that balanced helicase and exonuclease activities and structural support might be equally important for normal WRN function during NHEJ.

The results demonstrated that BLM suppresses HR repair, whereas WRN modestly enhances HR repair. Similar to the NHEJ assay, the HR repair assay suggested that the double mutant (E–H–) WRN cells exhibited HR repair frequencies similar to those of wtWRN cells. Interestingly, cells with either single WRN mutation (E– or H–) exhibited higher frequencies of HR repair than those of wtWRN or double mutant cells, but intermediate levels of NHEJ. One possible explanation for this is that because WRN single mutants are unable to restore NHEJ completely, there is a compensatory increase in HR repair by other proteins. This hypothesis implies that the primary function of WRN is to optimize DSB trimming of DNA during NHEJ. Alternatively, HR repair frequencies may be lowest in WRN–/– cells because their extensive deletion phenotype simply increases the probability of eliminating sequences homologous to nearby sequences, which would optimize HR repair. In E– or H– cells, unbalanced WRN activity might increase the accessibility of sequences that facilitate HR. However, because the preferred substrates for WRN are complex DNA structures (Fry & Loeb, 1999; Constantinou *et al*., 2000; Huang *et al*., 2000), this hypothesis seems less likely.

We found that BLM suppresses HR during DSB repair, as expected from the cytogenetic phenotype of BS cells. Although our experiments were not designed to compare directly the effects of BLM and WRN on HR repair, the differences in HR repair frequencies were higher in BLM–/– cells than in WRN–/– cells. These results are in agreement with the finding that BLM is the dominant factor that regulates HR in avian cell lines (Imamura *et al*., 2002).

In summary, our findings from cells that express mutant forms of WRN reveal a structural role for WRN in DSB repair, one that may involve protecting broken DNA ends from degradation and/or recruiting other proteins that mediate DNA repair. Our conclusions are indirectly supported by the fact that most of the *WRN* mutations identified in WS patients are premature terminations that lead to a loss of WRN nuclear localization, rather than mutations that eliminate WRN exonuclease or helicase activity. However, it should be noted that our studies were limited to exploring the role of WRN in DNA DSB repair. WRN may also participate in other DNA processes, such as regulation of transcription, replication and telomere function. As other potential functions of WRN are elucidated, it will be of considerable interest to compare the activities of WRN mutants to those of wtWRN in these processes. The helicase and exonuclease activities of WRN may play differential roles in other WRN functions, possibly

regulated by different interacting partners and/or post-translational modifications.

Experimental procedures

Cell lines

WS fibroblasts, 78-26, carrying a homozygous mutation at IVS25-1G > C, which causes exon 24 to be skipped and prematurely terminates the protein (Yu *et al*., 1996), were immortalized by the catalytic subunit of human telomerase (hTERT), as described previously (Gray *et al*., 1997). The full-length wild-type or various mutant human WRN cDNAs were retrovirally introduced into hTERT-immortalized WS cells. The mutants included the E84A mutation in the exonuclease domain (Huang *et al*., 1998), the K577M mutation in the helicase domain (Gray *et al*., 1997), or both, thereby generating the wtWRN, E–, H– and E–H– lines. BS fibroblasts, HG2654 cells (provided by Dr James German, Cornell University) carrying a homozygous mutation, c2006-2212delATCTGA, insTAGATTC, were immortalized with hTERT (Gray *et al*., 1997) to generate the BLM–/– hTERT cell line. The wild-type BLM cDNA was then introduced using a retrovirus to generate a complemented BS line. hTERT-immortalized 82-6 control cells have been described (Oshima *et al*., 2002). Cells were cultured in Dulbecco's modified eagle medium (DMEM), with 10% fetal bovine serum (FBS), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin at 37 °C in a 5% CO_2 atmosphere.

Western blot analysis

Western blotting was performed as described previously (Oshima *et al*., 2002). Briefly, samples containing 100 μg of total cellular protein were separated on 4–15% SDS-polyacrylamide gels and transferred to a nylon filter. WRN immunoreactivity was measured by incubating the filters with rabbit polyclonal antihuman C-terminal WRN and peroxidase-conjugated antirabbit antibodies, and followed by chemiluminescence detection.

V(D)J recombination assay

The V(D)J recombination assay was performed essentially as described previously (Yeo *et al*., 2000). Briefly, assay plasmids pGG49 or pGG51, and RAG1 or RAG2 plasmids were co-transfected into human cells at a ratio of 1 : 3 : 3 using Superfect (Qiagen) at a ratio of 6 : 1 (Superfect to DNA). Plasmids were recovered 2 days later for WS lines and 3 days later for BS lines. Plasmids were resuspended in 5 μL of water, and 1–2 μL of the plasmid solution was electroporated into *E. coli* strain MC1061. The bacteria were cultured on LB plates containing carbenicillin (Cb), or Cb + chrolamphenicol (Cm) at a ratio of 1 : 10. Plasmids were isolated from colonies that grew on Cb + Cm plates, and sequenced to confirm V(D)J cutting and rejoining. A minimum of 6000 colonies were counted in each of triplicate experiments.

PBS joining assay

As previously described (Oshima *et al*., 2002), pBluescript SKII (Stratagene, La Jolla, CA, USA) was digested with various combinations of restriction enzymes to produce a linear plasmid with non-compatible ends. *Eco*RI was used to create 5′ protruding ends, *Sma*I created 5′ or 3′ blunt ends, and *Sac*I created 3′ protruding ends. Linearized plasmids were transfected into human cells using Fugene6 (Roche Pharmaceuticals Co., Nutley, NJ, USA) according to the manufacturer's recommended protocol, using a Fugene6 to DNA ratio of 6 : 1. The transfected cells were maintained in DMEM with 10% FBS for 2 days. The cells were harvested by scraping and the plasmids were isolated and electroporated into *E. coli*. Sixteen bacterial colonies from each transformation were analysed by digestion with *Eco*RV and *Xmn*I to verify successful linearization. The constructs were then subjected to DNA sequencing to determine the extent of deletion at each end. The average number of nucleotides deleted in all 16 plasmids was calculated, and this was reported as the deletion size, as described previously (Oshima *et al*., 2002).

pCMS-homo assay

pCMS-homo was digested with *Eco*RI and *Xho*I to generate incompatible ends, and transfected into human cells using Fugene6, as described in the pBS joining assay. After 2 days, cells were trypsinized, collected and resuspended in DMEM for subsequent flow cytometric analysis. Cell fluorescence was analysed using two filters, 575/26 for enhanced yellow fluorescent protein (EYFP) and 510/20 for green fluorescent protein (GFP), and a fluorescence cytometer. Particles of the appropriate size for fibroblasts were gated for the scattering plot. The scattering plots were subjected to quadrant analysis using the SUMMIT program (Cytomation Inc., Fort Collins, CO, USA), and gated with mock, GFP, and EYFP, to determine the proper cut-off value for the analysis.

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